VIRO- BLOT
ANTI - BORRELIA - IgG/-IgM

Instruction for use

GB

1. Intended use

Immunoblot for qualitative determination of antibodies to Borrelia burgdorferi (Lyme) in human serum and plasma

IgG	REF	BG 111/24	⩔ 24	IVD	complete kit
IgG	REF	BG 111/8	⩔ 8	IVD	complete kit
IgM	REF	BM 111/24	⩔ 24	IVD	complete kit
IgM	REF	BM 111/8	⩔ 8	IVD	complete kit

2. Antigen Characterisation

Antigen: Borrelia burgdorferi whole cell antigen
Strain: Borrelia afzelii / Borrelia garinii / sensu stricto
Purification: Ultrasonication, centrifugation , filtration
Cultivation: Kelly-Medium

3. Introduction

Lyme disease (Lyme borreliosis) is a bacterial infection due to a tick-borne spirochete, *Borrelia burgdorferi.* This organism was first isolated from Ixodes dammini in the USA 1982, and later from Ixodes ricinus in Europe. Virtually all human cases of Lyme disease follow tick bite exposure.
Lyme disease is a multi-system disease, manifested in three stages and can occur in different organs (skin, joints, heart, eye, and nervous system) and either separately or in a combination of symptoms.

Stage I (Early): Appears after some days or a few weeks in the form of Erythema migrans accompanied by flu-like symptoms such as fever, vomiting, chills, headache and occasionally Lymphadenosis benigna cutis. At this stage IgM antibodies are present in 50-90% of patients. The prevalence of IgG antibodies is low, so that frequently a negative result is obtained for an IgG antibody determination.

Stage II: Becomes clinically apparent after some weeks or months. Cardiological complications as well as neurological disease e.g. meningitis or encephalitis may occur. (Cross over to multi-system disease). Antibodies to Borrelia burgdorferi are detectable in 70-90% of patients in this second stage. In the first weeks of the disease IgM is more prevalent, later IgG is more prevalent.

Stage III (Late): Months or years later some patients disease may progress to a third stage. In this stage arthritis, neurological disturbances, progressive encephalopathy or Acrodermatitis chronica athrophicans may develop. IgM antibodies are barely detectable in this late stage, while IgG antibodies are observed in 90-100% of patients.

Recovery is possible in Stages I and II even without treatment. However, due to the possibility of progression of the infection, antibiotic treatment of Lyme disease is recommended for all stages.

Kit content ⩔ 24 or ⩔ 8

NTS	24 / 8	**Nitrocellulose test strips** with purified, separated **B. afzelii/ garinii/s.s. proteins**
	3 / 1	**Incubation dish with** 8 chambers
WASHBUF 25x	25ml / 25ml	**Wash solution concentrate** (25x)
SPE DIL	2x25ml / 21 ml	**Dilution buffer** (with 0,05% microbial preservative) RTU
CONJ ALK 10x IgG IgM	2,8 ml / 1,0ml	**Anti-human IgG or IgM Alkaline Phosphatase** (sheep) (10x) (preservative: 0,049% Thimerosal)
CONTROL co 5x	1,0 ml / 0,4ml	**Cut-off control**, human (preservative: 0,095% sodium azid) (5x)
SUBS CH	25ml / 9ml	**Chromogen-/ Substrat solution** RTU
	1	**Evaluation diagram with positive Control strip IgG (optional)**
	1	**Protocol sheet**
	1	**extra sheet for BLOTrix software**

4. Principle of the WESTERN BLOT

The WESTERN BLOT may be used for the qualitative determination of human antibodies to Borrelia burgdorferi antigens.
For this Immunoblot two European strains of Borrelia burgdorferi (complete antigen) was treated with SDS and then electrophoretically separated in Polyacrylamide-gel according to molecular weight of the proteins. This was then transferred to Nitrocellulose (blotted) and cut into individual NTS.
The NTS were then incubated with blocking buffer.

For the assay the NTS are incubated with the control and the samples to be investigated, antibodies present will bind specifically to the protein bands and form antigen-antibody complexes. Unbound serum or plasma is removed by means of washing. During the second incubation Anti-human IgG or IgM Conjugate binds to the antibody-antigen complex. In the final wash step excess Conjugate is removed and SUBS CH substrate is added to the chamber.

The specific protein bands appear as purple bands. The reaction is then stopped by the addition of distilled water and the NTS may be dried.
Optional: With the aid of a kit specific evaluation diagram the band pattern may be assessed and evaluated.
The interpretation of the band pattern provides evidence of the antibody response.

5. Materials required, but not provided with the Kit

- Distilled water
- Pipettes
- Timer
- Measuring cylinder
- Tweezers
- Test tubes for sample- and conjugate dilution
- Water jet vacuum pump (to remove fluid from the incubation dish)
- Rocking platform or horizontal shaker

6. Precautions

The VIRO-BLOT test is for IVD use only.

1. Do not mix lot specific reagents, such as NTS, Controls and CONJ ALK 10x from different kit lots. The SUBS CH doesn't have to be from the original test kit. But the lot of the SUBS CH should be the same as indicated on the kit label. The SPE DIL, the WASHBUF 25x can be used for all VIRO-BLOT tests irrespective of the lot and the nature of the VIRO-BLOT.
2. Seal all bottles properly after use in order to avoid bacterial contamination. All samples and kit components should be considered potentially infectious. All control samples have been tested for Hepatitis Bs antigen, anti-HIV I and II , anti-HCV and found to be negative.
3. The NTS are coated with inactive antigen. However, normal laboratory precautions should maintained when handling with infectious material. Do not pipette by mouth.
4. Avoid contact with skin and mucous membranes when handling reagents, which contain preservatives (see kit contents). Wash thoroughly with water in case of contact and possibly look up a doctor.
5. Controls containing sodium azide may react with lead and copper plumbing, building explosive metal acids. Flush with sufficient water when disposing of reagents.
6. For disposal the legal regulations have to be followed.

7. Storage and stability

Store all reagents at 𝒴 2-8°C, protected from intense light. The expiration date of each component is indicated on the respective vial label. Do not use reagents beyond the expiration date.
The diluted WASHBUF is stable up to 4 weeks when stored at 𝒴 2-8°C.

8. Specimen collection and general handling

1. The assay procedure should be carried out only by qualified and well trained employees.
2. The instruction for use describes the applicable test method. In case of modification or applications others then the intended use, the user has to validate the procedure and take the responsibility for it.
3. Lipaemic, icteric, haemolysed or microbially contaminated specimen may cause interference.
4. Suitable specimens are serum or plasma (heparinized, EDTA, Citrat) samples obtained by standard laboratory techniques.
5. The samples should not be heat-inactivated since non-specific results may occur. Avoid repeated freeze-thaw cycles.
6. For ethical reasons, the use of CSF samples was only evaluated by testing pairs of CSF and serum samples (only if available), distributed by the "WHO Collaborating Centre of Quality Assurance and Standardization in Laboratory Medicine" in Germany. Therefore the use of CSF should be proved by testing a sufficient number of samples.
7. Patient samples should be stored at 𝒴 2-8°C.
   For long term storage 𝒴 -20°C or lower is recommended.
8. **Note:** Diluted patient samples must be used on the same day.

9. Preparation of the test

Bring all reagents to room 𝒴* prior to use !

* room 𝒴 up to maximally 25°C

WASHBUF : Dilute the WASHBUF 25x 1:25 with distilled water
e.g. add 10ml of WASHBUF 25x to 240 ml distilled water and mix well.

Control: Dilute the CONTROL co 5x **1:5** with Specimen diluent.
(800 µl Specimen diluent + 200 µl cut-off Control)

Dilution of samples: Dilute patient samples **1:51** with SPE DIL
e.g. 20µl sample + 1000µl SPE DIL; mix thoroughly.

Conjugate dilution: Dilute the CONJ ALK 10x during the first wash step with
SPE DIL e.g. for 5 strips: 4,5ml SPE DIL
+ 0,5ml CONJ ALK 10x = 5ml Conjugate dilution

Please note:

- Carefully remove the NTS using tweezers, holding the strip by the numbered end.
- Close the tube immediately after taking the NTS.
- The NTS should be used in number order from low to high.
- Lay the required number of NTS, one per chamber of the incubation dish, placing them with the number facing up.
- Ensure that the NTS remain covered with fluid and that they do not dry out between the incubation or wash steps.

The CONTROL co have to be used for every run IgG and IgM determination.
The cut-off control band in IgG and IgM is always detected as a weak band, the developed sample bands interpreted in relation to the intensity of this band of the cut-off control. Only more strong (positive) bands than the cut-off control will be evaluated.

10. WESTERN BLOT Assay procedure

Incubation at **room** *T* *****

- **First incubation (sample incubation) 60 min.**
  Carefully pipette 1000 µl diluted Control or 1020 µl pre-diluted patient sample into the incubation chamber to coat the strip and incubate **60 min.** on the rocking platform.
- **First wash step**
  Completely remove patient samples (with the vaccum pump) and wash each strip **1 x** with **2ml** prepared WASHBUF and immediately drain. Next wash the NTS a further **3 x** with **2 ml** WASHBUF for **5 min.** each time. (Incubation on the rocking platform).
- **Second incubation (conjugate incubation) 30 min.**
  Completely remove the WASHBUF and pipette **1 ml** pre-diluted CONJALK in each chamber.
  Incubate for **30 min.** on the rocking platform.
- **Second wash step**
  (see first wash step**)**
- **Third incubation (Substrate incubation) 15 min.**
  Completely remove the WASHBUF and pipette **1 ml** RTU SUBSCH in each well. Incubate from 10 min. to 15 **min.** on the rocking platform. Remove the SUBSCH completely from each compartment.
- Stop the enzyme-substrate reaction with **2x** 2 **ml** for 5 min. distilled water each.

Remove the NTS carefully from the compartment and dry completely on filter paper. The protein bands may be identified and evaluated with aid of the Evaluation diagram.

For the purposes of documentation the NTS should be kept dry and protected from the light.

11. Test results

Every NTS has two controls: a **serum control** and a **conjugate contro**l (IgG / IgM separat). If the assay handled right, you can see on top of the NTS the purple coloured control bands of both (see control strip) .
The test procedure is valid, if serum control as well as the conjugate control appears claerly visible on the developed strip. In case not only one conjugate control band appears, the more reactive gives notice to the used conjugate. Refer to the kit specific positive control strip card for information of the exact position of the serum- and conjugate control.

Usage/evaluation of the cut-off control:
Cut-off band IgG: VlsA
Cut-off band IgM: p25
Bands with an intensity weaker than the cut-off control are not considered for the interpretation.

The proteins p41 and p39 are only high specific for IgM.
The width of the bands as well as their intensity are variable and depend on the concentration of the detected Borrelia antibodies in the sample.
The band pattern in the IgG determination is generally more intense and diverse than IgM.

Evaluation of the strips manuell:
Match the dry NTS at the edge of the protocol sheet and fix the NTS with tape or similar at the numbered ends of the NTS.
The identification of the bands is possible with the kit specific positive control strip card. The high specific bands are signed on the positive control strip card.
The control card will be matched at the incubated fixed patient NTS, the black line have to agree.
Now it is possible to read directly the immuno specific bands.
Record the recognisable bands according to their molecular weight and intensity (always in consideration to the co control) on the protocol sheet.

Interpretation of result by using BLOTrix software from VIRO-IMMUN
The result of a NTS can be interpreted by using the BLOTrix software.
Evaluation mode is described in the user manual.

Borrelia immune reactive proteins

Table 1

Molecular weight In kDa	Antigen	Specificity
>101		not specific
(92-100) **p100**	*Borrelia surface membrane vesicle protein*	**high specific** (late) in VIRO-BLOT
p83-91	*breakdown products*	specific (late)
p59 / p62 complex	*heat shock protein membrane protein*	**highly specific** in VIRO-BLOT
p45	*Important IgG marker*	specific
(p41-43) **p41** (p39-40) **p39**	*(Flagellin protein) associated with Flagellin-complex*	Not specific, cross- reactive, a heavy band may indicate a Borrelia infection (**highly specific** for **IgM**)
p36-38		probably specific
p35	*Osp B external membrane protein*	**high specific**, detectable at all stages of the infection, patients often show only a weak reaction
p31	*Osp A*	Specific man-made, less sensitive serological marker
p30	Frequent positive IgG marker in stage II and III	**highly specific** in VIRO-BLOT
p28/29		probably specific
(p23-26) **p25**	*Plasmid coded ipoprotein Osp C IgM Marker in stage I +II*	**high specific** in VIRO-BLOT, important and most common IgM marker
p20 / p21 complex	Frequent positive IgG marker in stage II and III	**high specific** in VIRO-BLOT,
P17 / p19 complex	Plasmid-coded Osp important IgG marker	**high specific** in VIRO-BLOT, (conserved within B. afzelii)
p18 kDa		genus specific

early - acute infection - more frequent detection of IgM antibodies

late - chronic infection - more frequent detection of IgG antibodies, rarer detection of IgM antibodies

12. Diagnostic Relevance and interpretation of results

Serological findings in a Borrelia infection depend on the stage and duration of the disease, the severity of the symptoms as well as previous antibiotic therapy. To obtain a final diagnosis the patient history and clinical symptoms should be taken into consideration.

IgM antibodies are primarily detectable in Stage I, although some patients remain IgM negative. In the case of long disease duration and in the late stage almost all detectable **antibodies are IgG**. A sharp increase in IgG antibodies is observed during reinfections. Where neurological symptoms are present CSF should be examined in addition.

BORRELIA IgG:

Both the intensity and position of bands should be taken into consideration when assessing VIRO-BLOT bands.
p41 and 39kD is certainly a genus specific antigen protein, but p41 is also cross-reactive and thus to be considered with care.
Impotant: Only one band in a complex will be considered for the result.
Exclusion: double band p59 p62 are in combination more positive.

Bands occurring:

highly specific and specific antigen bands
p100kD/ p59 u 62kD complex/ p45kD / p35kD/ OspA / p30kD
p25kD / p21 u p20kD complex / p19 u p17kD complex

highly specific and antigen band
VlsA

Recommended BORRELIA IgG interpretation:

negative	- a weak antigen - 1 positive band - only one positive band from p59 p62 complex - no or very weak band VlsA
borderline/ ***questionable***	- a positive antigen double band from p59 p62 - 1 heavy antigen band - 2 positive bands - 1 positive band and at least 2 further weak bands (= co control) - weak VlsA band, intensity = VlsA co control
positive	- positive VlsA band, intensitiy > co control - a positvs double band p59 p62 and at least 1 further weak band - at least 3 positive antigen bands - at least 1 heavy band and at least 1 positive band - at least 2 heavy bands

In patients in a late phase of Borrelia infection particularly, many antigen bands from classes A and B may be observed.

BORRELIA IgM:

Both the intensity and position of bands should be taken into consideration when assessing VIRO-BLOT bands.

Although p41kD is a genus specific antigen protein and is cross-reactive, in VIRO-BLOT a p41 and 39kD band reaction should be judged for Borrelia IgM as a positive reaction.

IgM antibodies, which are considered early markers of Borrelia infection, are frequently observed against Flagellin and Osp C.

Bands occurring:

highly specific antigen bands
p41kD/ p39kD/ p21 u p20kD complex/ p19 u p17kD complex

Recommended BORRELIA IgM interpretation:

negative	- no antigen bands - 1 weak antigen band
questionable	- 2 weak antigen bands
positive	- at least 1 positive antigen band , particulary for Flagellin (p41 or p39) and Osp C (p25)

13. Performance Characteristics

Specificity / Sensitivity

216 samples IgG and 62 samples IgM were tested parallel in VIRO-BLOT Anti-Borrelia IgG and IgM and comparison methods ELISA.
The sensitivity and specificity are based on the results found.

Specificity: IgG 97,9% **Sensitivity:** IgG 87,8%

Specificity: IgM 100% **Sensitivity:** IgM 82,6%

Borderline results were not taken into account in the calculation.

14. Cross-reactivity

- In the early infection phase there may be an insufficient number of antibodies and therefore a negative test result is possible.
- When there is a clinical suspicion of Borrelia infection with a negative or questionable test result, a repeat test two weeks later is recommended.
- Antibiotic therapy may suppress the production of detectable antibodies in the early stages.
- Despite the presence of detectable IgG antibodies an active Borrelia infection need not be present, since IgG antibodies may persist for a long time after a previous infection.
- Leptospira and Treponema infections are known to show cross-reaction with Borrelia antigens.
- An acute EBV infection may lead to the stimulation of Borrelia antibodies. In the case of an unclear clinical picture this infection should be excluded with appropriate tests.
- The serological data should be taken into consideration with the clinical history for a full diagnosis. Where the results are questionable repeated testing is recommended.

15. Precision and reproducibility

Intra-assay reproducibility was determined by testing samples of different levels of antibody activities in one test run.
In determination of the samples there is no significant difference of detected antigen bands and their intensity (constant level).
Inter-assay reproducibility was determined by testing samples of different levels of antibody activities in different test runs.
In determination of the samples there is no significant difference of detected antigen bands and their intensity (constant level).

16. Internal quality control

The cut-off control should be carried out with every test run.
Recommendation: For every new Lot, it is recommended to perform an internal quality control by testing one or more internal control samples which should be clearly specified.

17. Literature

- Karlsson, M. J. Clin. Microbiol., 28 (5), 2148-2150
- WilskeB, .. FEMS Immunol Med. Microbiol. 291(2007) 13-21,

18. Troubleshooting

ERROR	POSSIBLE CAUSES
no colourimetric reaction after addition substrate	no CONJ ALK pipetted, contamination of CONJ ALK (possibly with control sera during pipetting) may cause an inactivation.
generally too high reaction	incorrect CONJ ALK (i.e.not from original test kit), incubation time too long or incubation temperature too high, water quality for WASHBUF insufficient (low grade of deionization)
generally too low reaction	incorrect CONJ ALK (i.e.not from original test kit), incubation time too short, incubation temperature too low
false positive / negative samples	incorrect dilution of samples, lipaemic, haemolytic or icteric samples
unexplainable outliers	contamination of pipettes, tips or containers or with metals (iron, copper etc.),
high variation (from series to series)	incubation conditions not constant (time, temperature) high variation of room temperature, controls and samples are not carried out at same time (same intervals) check pipetting order, person related variation.
generel source of error, that may leads to incorrect results	- surface damaging of NTS because of improper treatment - NTS dried out after washing (unreproducible results). - Reagents not pre-warmed to room temperature prior to use(NTS)!. - Insufficient washing

The close observance of the test procedure will help to avoid these errors

WESTERN BLOT SHORT ASSAY PROCEDURE

Dilute **patient sample 1:51 :**
20 µl patient sample + 1000µl SPE DIL.
Dilute **controls 1:5:**
800µl SPE DIL + 200 µl control
Dilute **CONJ ALK 10x 1:10:**
f.e: 4,5ml SPE DIL + 0,5 ml CONJ ALK 10x

Take required NTS holding with tweezers by the numbered end out of the tube.
All inkubations / wash steps on the rocking platform

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PIPETTE 1 ml DILUTED SAMPLES /
DILUTED CONTROLS EACH NTS AND CHAMBER

▼

FIRST INCUBATION 60 MIN.

▼

FIRST WASH STEP

▼

After incubation wash **4 times** with WASHBUF

▼

Pipette **1000 µl** CONJ ALK each chamber

▼

SECOND INCUBATION 30 MIN.

▼

SECOND WASH STEP (see 1. Wash step)

▼

Pipette **1000µl** RTU SUBS CH each chamber

▼

THIRD INCUBATION / SUBSTRATE INCUBATION
15 MIN.

▼

After **15 min** stop the Enzyme/Substrate reaction
2 times 5 min. with 2 ml aqua dest.

▼

VISUEL EVALUATION OR

WITH VIRO-BLOT SOFTWARE

: CE
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